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A Comparison of Glycine, Sarcosine, *N*,*N*-Dimethylglycine, Glycinebetaine and *N*-Modified Betaines as Liposome Cryoprotectants

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Abstract—Glycinebetaine has previously been shown to be effective at reducing leakage from liposomes which are frozen then thawed. This study involved the preparation of a series of *N*-modified betaines and the comparison of their cryoprotective activities with those of glycine, sarcosine, *N*,*N*-dimethylglycine and glycinebetaine. All the compounds investigated, with the exception of (dimethyloctylammonio)acetate, reduced the degree of leakage, after freezing and thawing, with additive concentrations up to 0·6 M. Reducing the degree of *N*-terminal methylation of glycinebetaine appeared to increase the leakage from liposomes at additive concentrations between 0·2 and 0·6 M. (Dimethylethylammonio)acetate, (dimethylisopropylammonio)acetate and (*N*,*N*,*N*′,*N*′-tetramethylethylenediammonio)-*N*,*N*′-diacetate appeared to be no more effective than glycinebetaine, whereas improved protection was afforded by (triethylammonio)acetate and (diethylmethylammonio)acetate at most concentrations. This study demonstrates that the cryoprotective activity of glycinebetaine may be improved with modifications to the *N*-terminal.

The use of liposomes for the pharmaceutical formulation of water soluble drugs is limited by the leakage and instability of the entrapped drug solution on storage. These problems could be reduced substantially by the use of frozen or freeze-dried liposome preparations. However, it is first necessary to develop suitable additives to reduce damage to liposomes on freezing, thawing and dehydration. It is well recognised that freeze-thaw damage can be reduced by the addition of cryoprotectants to the liposome suspension (Higgins et al 1986; Crommelin & Van Bommel 1984). These additives reduce intraliposomal ice formation and osmotic stresses on liposomes during the freezing and thawing processes. A wide variety of compounds has been shown to be effective as liposome cryoprotectants including glycerol, dimethylsulphoxide and various saccharides, but as yet no compound has been shown to prevent fully the leakage of an entrapped water soluble marker.

Glycinebetaine (betaine), a common plant metabolite, has also been shown to reduce freeze-thaw damage to liposomes (Higgins et al 1987). This cryoprotectant had previously been implicated in the protection of plants against salt stress (Storey & Wyn Jones 1977) and has been demonstrated to protect enzymes from salt damage (Pollard & Wyn Jones 1979). Wyn Jones et al (1977) proposed that betaine protects plants from salt stress by acting as a cytoplasmic osmoticum. Further studies have shown that the nitrogen atom of glycinebetaine must be fully methylated for activity (Sauvage et al 1983). The *N*-methylation of glycine increases the water solubility and hygroscopic nature so that glycinebetaine attracts and retains water within the cytoplasm more effectively than glycine (Le Rudulier et al 1984).

The purpose of this study was to investigate the effect of *N*-terminal modification on the ability of glycinebetaine to reduce freeze-thaw damage to multilamellar vesicles.

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Materials and Methods

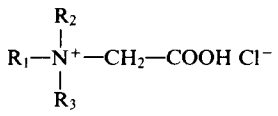
Preparation of N-*modified betaines*

Glycine, sarcosine (*N*-methylglycine) and *N*,*N*-dimethylglycine were obtained from Aldrich Chemical Company (UK) and were used without further purification. The other *N*-modified betaines were prepared by modification of the methods of Balle & Eisfield (1937), Linfield et al (1963) and Beckett & Woodward (1963). The betaines were prepared as hydrochlorides and converted to the corresponding inner salts by ion exchange chromatography. The structures of the hydrochlorides were confirmed by infrared spectroscopy, ^{1}H-NMR spectroscopy and elemental analysis (Table 1). The melting points are uncorrected. The microanalyses were performed on a Perkin-Elmer 240 by Butterworth Laboratories (Middlesex, UK).

Preparation of (carboxymethyl)trialkylammonium chlorides

(Carboxymethyl)dimethylethylammonium chloride (I), (carboxymethyl)diethylmethylammonium chloride (II), (carboxymethyl)triethylammonium chloride (III) and (carboxymethyl)dimethylisopropylammonium chloride (IV) were prepared by the following general method. Ethylchloroacetate (0·1 mol, 12·25 g) was refluxed with the appropriate tertiary amine (0·1 mol) in ethanol (25 mL) for 1 h. The ethanol was removed from the reaction mixture by evaporation under reduced pressure to give a white residue which was dissolved in 3·6% w/v HCl (100 mL). This mixture was refluxed for 3 h. Evaporation of the solvent under reduced pressure gave a white product which was recrystallized from an acetonitrile/water mixture. The (carboxymethyl)dimethyloctylammonium chloride (V) was prepared using a modification of this procedure. Chloroacetic acid (0·1 mol, 9·4 g) was neutralized with 10% NaOH solution. Dimethyloctylamine (0·1 mol, 20·5 mL) and ethanol (50 mL) were added and the mixture refluxed until the solution was neutral to litmus. Concentrated HCl (12 mL) was added and

Table 1. Structure and characterization of betaine hydrochlorides.



Compound	R_1	R_2	R_3	Melting point (°C)	Elemental analysis (theoretical) C	H	N
I	$-CH_2CH_3$	$-CH_3$	$-CH_3$	187–189	42·9 (43·0)	8·4 (8·4)	8·3 (8·4)
II	$-CH_2CH_3$	$-CH_2CH_3$	$-CH_3$	178–181	46·5 (46·3)	8·8 (8·8)	7·7 (7·7)
III	$-CH_2CH_3$	$-CH_2CH_3$	$-CH_2CH_3$	218–220	48·9 (49·1)	9·2 (9·2)	7·1 (7·2)
IV	$-CH(CH_3)_2$	$-CH_3$	$-CH_3$	207–208	46·3 (46·3)	8·9 (8·8)	7·9 (7·7)
V	$-(CH_2)_7CH_3$	$-CH_3$	$-CH_3$	161–163	57·3 (57·2)	10·3 (10·4)	5·5 (5·6)
VI	$-CH_2CH_2N^+(CH_3)_2CH_2COOH.Cl^-$	$-CH_3$	$-CH_3$	204–205	38·8 (39·3)	7·4 (7·2)	9·0 (9·2)

the mixture evaporated to dryness. The residue was extracted with ethanol, the cold extract filtered, and the filtrate evaporated to dryness and the product recrystallized from ethanol/ether.

Preparation of N,N′*-dicarboxymethyl-*N,N′*-tetramethyl-ethylenediammonium dichloride (VI)*

N,N′-Tetramethylethylenediamine (0·1 mol, 15 mL), ethyl-chloroacetate (0·3 mol, 30 mL) and ethanol (50 mL) were refluxed for 1 h. The ethanol was removed by reduced pressure rotary evaporation. HCl 3·6% w/v (100 mL) was added to the residue and the mixture refluxed for 3 h. Removal of water under reduced pressure gave a gelatinous solid which formed a white precipitate on addition of ethanol (100 mL). The white solid was collected by filtration using a sintered glass funnel and was recrystallized from ethanol/water.

The preparation of ω-(trialkylammonio)carboxylates from (ω-carboxyalkyl)-trialkylammonium halides using ion-exchange

Betaine hydrohalides are generally less hygroscopic and more readily prepared than their corresponding (trialkylammonio)carboxylates. The conversion of the hydrohalides to the (trialkylammonio)carboxylates is most readily achieved by passing an aqueous solution of the (ω-carboxyalkyl)trialkylammonium halides through an anion exchange column (Beckett & Woodward 1963). The chloride ion is exchanged for the hydroxide ion on the column and the inner salt recovered by evaporation of the water under reduced pressure.

A 300 × 25 mm glass column was packed with 100 g of anionic exchange resin (Dowex-1, 1X8-50, 8% cross-linked). The column was equilibrated by passing NaOH (2 M, 100 mL) through at a rate of 2 mL min^{-1}. The column was then rinsed with distilled water until the eluent was no longer alkaline to litmus (ca. 500 mL). The betaine hydrohalide (ca. 5 g) was dissolved in water (ca. 5 mL) and pipetted onto the column. A reservoir of distilled water was attached to the head of the column and the betaine eluted from the column at a rate of 2 mL min^{-1}. After collection of 200 mL of eluent, the water was removed by evaporation under reduced pressure to give the (trialkylammonio)carboxylate. Final traces of water were removed either by the addition and evaporation under reduced pressure of 25 mL of dehydrated ethanol or by freeze-drying the sample overnight in an Edwards Modulo freeze-drier. The dried (trialkylammonio) carboxylates were stored over phosphorus pentoxide in a vacuum desiccator until required.

Liposome preparation

A solution of egg lecithin (Lipid Products) dissolved in chloroform (50 mg in 25 mL), was deposited onto the walls of a 250 mL round bottom flask as a thin film, by reduced pressure rotary evaporation. Final traces of the solvent were removed by rotating the flask in a stream of oxygen-free nitrogen for one min. The lipid was hydrated with a 1% w/v solution of a water soluble dye, amaranth (BDH), in 0·02 M pH 7·0 phosphate buffer (5·0 mL). To assist the formation of the liposomes, three glass beads ($\phi = 5$ mm) were added and the flask rotated at atmospheric pressure in a waterbath at 50°C for 15 min. The liposome suspension was transferred to a 10 mL polypropylene tube and stored at −20°C until required. After thawing, the liposome suspension was freeze-thawed three times by direct immersion of the tube in liquid nitrogen followed by immersion in a waterbath at 50°C, to improve the entrapment. The resultant liposome suspension was maintained at room temperature (21°C) for 1 h to anneal the liposome structure. The suspension was then diluted with 0·02 M pH 7·0 phosphate buffer to give a lipid concentration of 2 mg mL^{-1} and the liposomes pelleted by centrifugation at 18 000 rev min^{-1} (30 000 *g*) for 10 min, 4°C (Sorvall, RC-5B centrifuge). The liposomes were washed twice to remove the unentrapped amaranth, before resuspension in 0·02 M phosphate buffer to give a stock solution containing 10 mg mL^{-1} lipid for use in freeze-thaw experiments.

Liposomes prepared in this way had a slight positive zeta potential (+7 mV, Coulter Delsa) when suspended in the buffer solution; they were multilamellar (electronmicroscopy) and had a particle size of 1–10 μm (Malvern Mastersizer). Of the amaranth solution added, 2·6% was entrapped giving an entrapment of 2·5 mg of amaranth per 100 mg of lipid.

Freeze-thaw protocol and determination of entrapped amaranth

The liposomes were diluted with cryoprotectant solutions and aliquots of the liposome suspensions (200 μL) were placed in 1·5 mL polypropylene Eppendorf centrifuge tubes. Samples were frozen in a circular rack by immersion into

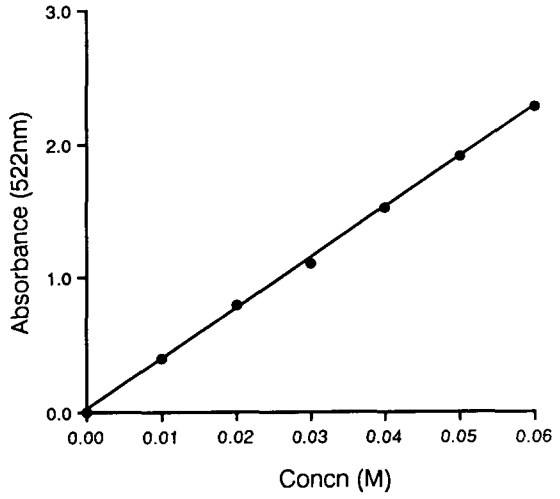


FIG. 1. The calibration curve for the spectrophotometric determination of amaranth in 50% propan-1-ol solution. Coefficient of correlation = 0·999.

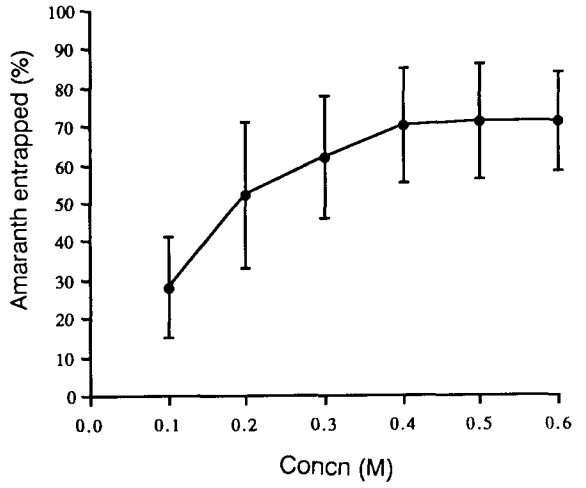


FIG. 2. Effect of glycinebetaine concentration on the amaranth loss from various batches of liposomes frozen then thawed. Bars indicate s.d. (n = 10).

liquid nitrogen (2 min) and then thawed in a waterbath at 50°C (2 min). Phosphate buffer (1·0 mL) was added to each tube and the liposomes pelleted by centrifugation at 15000 rev min^{-1} (15600 *g*) in an Eppendorf centrifuge (model 5414S). The supernatant was discarded and the liposome pellet washed by resuspension in buffer and further centrifugation. The liposome pellets were finally solubilized in 50% propan-1-ol (1·2 mL). The amaranth remaining entrapped was determined by measuring the absorbance of each solution at 522 nm using a Perkin-Elmer Lambda 2 spectrophotometer. A calibration plot for the assay of amaranth in semi-micro glass cuvettes is shown in Fig. 1.

The cryoprotective activity was expressed as the percentage of amaranth remaining entrapped after the sample had been freeze-thawed.

Determination of freezing and thawing rates

The rates of freezing and thawing were measured using a system consisting of two copper-constantan thermocouples (fast response, 0·19 mm diam., T76/1 Comark (BS4937 (1974))) with their constantan leads joined together. The reference thermocouple was placed in ice/water and the sample thermocouple was placed in a freezing tube containing a sample of the liposome suspension. The copper leads were connected via an interface to a BBC microcomputer which was then able to record the changes in electrical potential with temperature. The system was calibrated using the freezing points of different solvent systems. The rate of freezing (between −20 and −50°C) on immersion into liquid nitrogen was 720°C min^{-1} and the rate of thawing in a waterbath at 50°C over the same temperature span was 840°C min^{-1}.

Results and Discussion

Samples of amaranth-containing liposomes from various batches suspended in buffer alone or buffer with various concentrations of glycinebetaine were subjected to the freeze-thaw protocol outlined above to determine the effects of

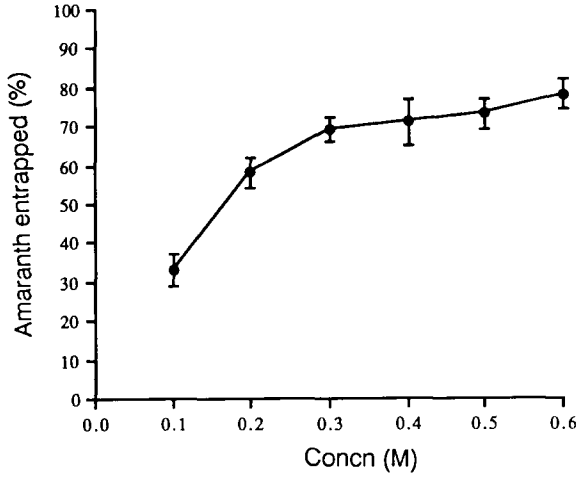


FIG. 3. The effect of glycinebetaine on amaranth loss from liposomes from the same batch frozen then thawed. Bars indicate s.d. (n = 4).

batch variation on the liposomal cryoprotection (Fig. 2). The percentage of amaranth lost from the liposomes at all concentrations of glycinebetaine varied markedly amongst batches. The reproducibility of using liposomes from the same batch for each determination was also investigated (Fig. 3). The variability, indicated by the standard deviations, between separate determinations at the same concentration was less marked using the same batch of liposomes. For comparisons amongst different compounds liposomes from the same batch were used for each assay. To facilitate this, small batches of liposomes were pooled before flash freezing.

Triplicates of amaranth-containing liposomes suspended in buffer alone or buffer with various concentrations of the various betaines were freeze-thawed as outlined above. In control experiments, in which the liposomes were not frozen, there were no differences in leakage over the duration of the experiment. Fig. 4 shows the effect of freezing liposome samples in the presence of various concentrations of glycine,

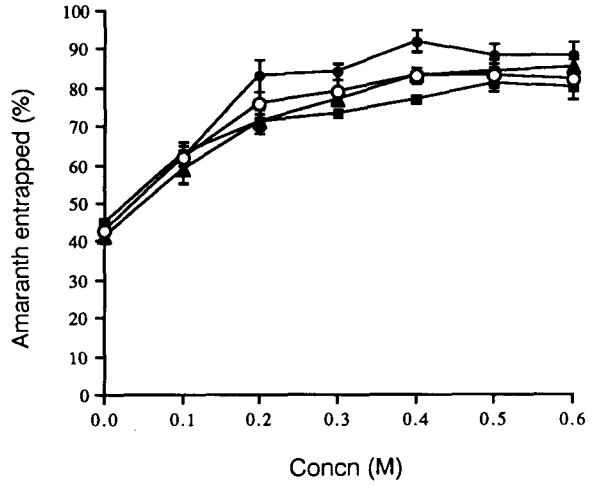


FIG. 4. The effect of glycinebetaine (●), *N,N*-dimethylglycine (○), sarcosine (▲) and glycine (■) on amaranth loss from liposomes frozen then thawed. Bars indicate range of values (n = 3).

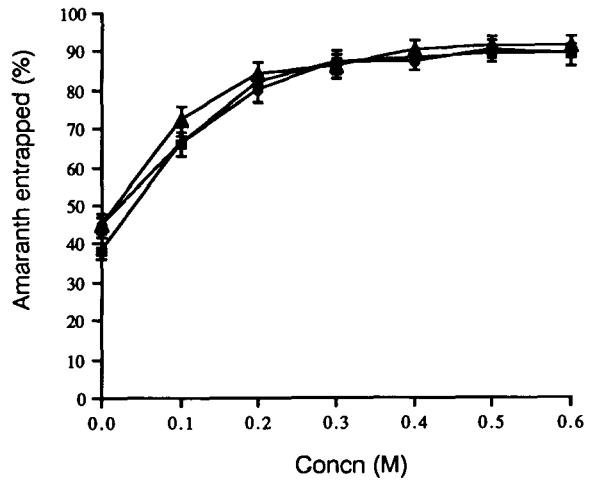


FIG. 6. The effect of glycinebetaine (●), (diethylmethylammonio)acetate (▲) and (dimethylethylammonio)acetate (■) on amaranth loss from liposomes frozen then thawed. Bars indicate range of values (n = 3).

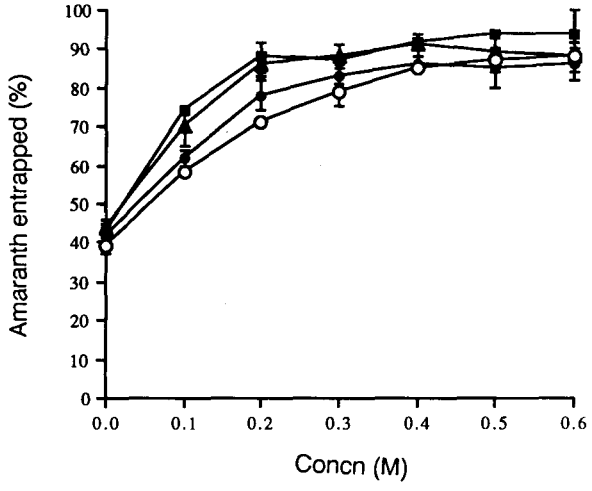


FIG. 5. The effect of glycinebetaine (●), (*N,N,N′N′*-tetramethylethylenediammonio)-*N,N′*-diacetate (○), (dimethylisopropylammonio)acetate (▲) and (triethylammonio)acetate (■) on amaranth loss from liposomes frozen then thawed. Bars indicate range of values (n = 3).

sarcosine, *N,N*-dimethylglycine and betaine. The amaranth leakage decreased with additive concentration in each case.

Figs 5 and 6 show the results obtained for the other *N*-modified betaines. There appears to be some increase in the retention of amaranth within the liposomes on freezing in the presence of the (dimethylisopropylammonio)acetate and the (triethylammonio)acetate at most concentrations. However, in the presence of (dimethylethylammonio)acetate, (diethylmethylammonio)acetate and (*N,N,N′,N′*-tetramethylethylenediammonio)-*N,N′*-diacetate there appeared to be no improvement on that observed for glycinebetaine. The leakage caused by these compounds in the absence of freezing was very small. (Dimethyloctylammonio)acetate, not shown, caused an increase in leakage of amaranth on freeze-thaw.

The mechanism of action of glycinebetaine in salt stressed plants has been attributed to either an interaction with water in the cytoplasm or to a direct membrane interaction (Jolivet et al 1982; Le Rudulier et al 1984). The ability of glycinebetaine to reduce leakage from liposomes on freeze-thaw may also be attributed to these mechanisms. The results of this study suggest that decreasing the *N*-terminal methylation of glycinebetaine reduces the ability of these compounds to prevent leakage of an entrapped material on freeze-thaw. Rudolph et al (1986) have already suggested that glycinebetaine acts by some kind of intercalation between the phospholipid head groups. If this is correct the results from this study would suggest that the interaction between the compounds and the membranes increases with the *N*-terminal alkylation of glycine suggesting that increasing the bulkiness of the *N*-terminal increases the interaction between the betaines and the phospholipid headgroups in the membrane. However, if the *N*-terminal alkyl chains are too long the betaines would appear to disrupt the phospholipid membrane thereby increasing leakage on freeze-thaw. However, an alternative mechanism, involving the interactions of betaines with water, may support the results of this study more readily. Visual observations of the freezing of liposomal suspensions have shown that extensive ice formation occurs on freezing liposomes in buffer alone. This results in the packing of the liposomes into the centre of the tube. On freezing in the presence of the additives the liposomes appear to remain more dispersed in the frozen medium suggesting that glass formation has occurred. The ability of these additives to reduce leakage from liposomes may therefore be due to the ability of these compounds to promote glass formation. Decreasing the methylation of glycinebetaine would therefore be regarded as reducing the ability of the compound to promote glass formation whereas increasing the bulkiness of the *N*-terminal helps promote glass formation.

The ability of a compound to promote glass formation is related to the way in which the compound structures water. Tyrrell & Kennerley (1968) showed that glycine had a primary solvation sheath centred on the charged nitrogen

and that this solvated solute destructured bulk water whereas glycinebetaine which has a lower electrostatic potential at the van der Waal's surface surrounding the nitrogen, did not appear to interact in any way with the solvent. The ability of these compounds to reduce leakage may therefore be correlated with the electrostatic potential at the van der Waal's surface around the amine moiety, which decreases with increasing bulkiness, as well as with their ability to interact with the liposome surfaces.

At present it is difficult to fully elucidate the mechanism of action of this class of cryoprotectants, however, this study does clearly demonstrate the importance of the *N*-terminal alkylation of glycine in minimizing the degree of leakage of entrapped water soluble drugs from liposomes on freeze-thaw.

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